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Original Paper

Time Course of Methotrexate Polyglutamate Formation and Degradation in the Pre-B-leukaemia Cell Line Nalm6 and in Lymphoblasts from Children with Leukaemia

B. Buchholz,[1] E. Frei,[2] J. Eisenbarth,[3] M. Weigand[2] and R. Ludwig[4]

[1]Children's Hospital, Klinikum, Mannheim, Mannheim; [2]Molecular Toxicology; [3]Radiochemistry, German Cancer Center, Im Neuenheimer Feld 280, D-69120 Heidelberg; and [4]Children's Hospital, Section Oncology, University of Heidelberg, Germany

With the aim of investigation, the mechanisms of resistance to methotrexate (MTX) in children refractory to leukaemia-treatment, we established a method of analysing MTX metabolism in Nalm6 cells (human pre-B). The optimal extracellular concentration for MTX uptake and MTX polyglutamate (MTXPG2-6) formation at a density of 5×10^6 cells/ml was 1 μM ^{3}H-MTX. After 15 h incubation at this concentration, a plateau of 5 pmol/10^6 cells of total MTX accumulated in the form of equal amounts of polyglutamates 3, 4 and 5 and low amounts of MTX and polyglutamates 2 and 6. MTX preloaded cells rapidly lost MTX and MTXPG2 in MTX-free medium, while MTXPG5 was still formed and then degraded very slowly. After 8 h in medium without MTX, 40% of total MTXPG was lost, after 24 h, 70%. The method is feasible for patient blasts. The number of blasts isolated from bone marrow after diagnosis is enough to perform small kinetic studies. The uptake of MTX into patient blasts is about 1/10 of that in Nalm6 cells.

Key words: methotrexate metabolism, polyglutamates, leukaemia cell line, lymphoblasts, resistance

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INTRODUCTION

DRUG RESISTANCE of cancer cells is the major reason for treatment failure in cancer patients treated with chemotherapeutics. Methotrexate (MTX) is included in all therapy protocols against common acute lymphoblastic leukaemia (ALL) and non-Hodgkin's lymphoma in children [1]. Currently, 70% of afflicted children are completely healed by polychemotherapy [2]. The therapy of children with relapse is difficult because the lymphoblasts become resistant to the chemotherapeutics used, probably including MTX [3].

MTX and its metabolism is one of the best-studied models for drug resistance (reviewed in [3]). The carrier for the reduced folates actively transports MTX into the cell [4]. In the cytoplasm, MTX is a substrate for the enzyme folylpolyglutamate synthase which converts folates and also methotrexate to their polyglutamates with up to 6 γ-glutamyl residues (MTXPG2-6) which, like the folate polyglutamates, persist longer intracellularly than free MTX [5]. The major cytostatic effect of the MTXPGs depends on the ability to bind to and inhibit, among others, the enzymes dihydrofolate reductase [6] and thymidylate synthase, which are both necessary for DNA synthesis. MTXPG2-5 inhibit thymidylate synthase much more efficiently than MTX, with a Ki decreasing from 13 μM for MTX to 0.17 μM for MTXPG2 and 0.047 μM for MTXPG5 [7]. Therefore, the capacity of a cell to form long chain MTXPG directly enhances the inhibition of thymidylate synthase.

Many mechanisms of drug resistance have been studied in cultured cells, but only a few could be proven in cancer cells from patients (reviewed in [8]). Resistance to MTX may be caused by decreased MTX uptake [9, 10], altered dihydrofolate reductase ([11], reviewed in [9]) and thymidylate synthase [12] activities, and reduced synthesis of MTXPG [13]. In cells made resistant to MTX *in vitro*, all mechanisms of resistance can occur simultaneously [14]. In

Correspondence to E. Frei.
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fresh tumour samples, dihydrofolate reductase gene amplification or mutation has only been observed in 3 patients [8], while it is very common in cells made resistant to MTX *in vitro*. A recent study using fluorescent MTX in ALL patients discovered two populations of blasts with different amounts of dihydrofolate reductase [11]. Decreased MTX uptake has been shown as a cause of MTX resistance [9]. To date, it is still unclear whether one mechanism or a combination of several mechanisms is responsible for acquired or inherent drug resistance in children with leukaemia or lymphoma refractory to treatment. Studies on MTXPG formation in freshly isolated blasts have shown MTX uptake and MTXPG formation to be important factors in resistance to MTX [15–18].

To study the role of MTXPG in the efficacy of chemotherapy with MTX in more detail, we developed a method to analyse the kinetics of MTX uptake, MTXPG formation and MTXPG persistence. The development of the methodology was performed with Nalm6 cells. These cells were isolated from a 19 year old boy originally diagnosed with non-B non-T ALL at relapse, and later classified as pre-B-cell leukaemia by surface antigens [19]. Since 80–95% of all childhood ALL develop from B-cell precursors [2], this cell line was considered a good model for fresh patient blasts. The established method was then validated with blasts from 2 patients, where a sufficient number of cells could be isolated from bone marrow to perform kinetic studies.

PATIENTS AND METHODS

Bone marrow was aspirated from patients for diagnosis. For the study reported herein, bone marrow remaining after diagnosis from 2 patients was used. 1 patient was a boy of 7.5 years in whom a common acute lymphoblastic leukaemia (c-ALL) was diagnosed, the other patient was a boy of 5.5 years with T-cell derived acute lymphoblastic leukaemia (T-ALL). Both patients are in remission.

Processing of patient samples

Blasts were isolated, after diluting the marrow 1:5 in phosphate buffered saline without Ca/Mg and with 1% fetal calf serum (PBSS), by standard Ficoll-Hypaque density centrifugation [20], washed twice in PBSS and viability assessed by trypan blue exclusion. Blasts were isolated and processed within 24 h after bone marrow aspiration.

Drugs and chemicals

MTX/MTXPG1 and 2,4-diamino-N^{10}-methylpteroic acid were gifts from Lederle Co., Wayne, U.S.A.; MTXPG2-4 were gifts from B. Chabner, Bethesda, Maryland, U.S.A. and MTXPG5-7 were bought from Schircks Co., Jona, Switzerland. 7OH-MTX was isolated according to Breithaupt and Künzlen [21] from urine of children treated with MTX. [3′,5′,7-^{3}H]-MTX with a specific activity of 17–24 Ci/mmol was bought from Amersham Co., Braunschweig/Germany or Moravek Biochemicals, Brea, U.S.A. Purity of ^{3}H-MTX was determined by high performance liquid chromatography (HPLC) as described below and ^{3}H-MTX was diluted with MTX to 5 Ci/mmol for the experiments. All other chemicals were purchased at the highest purity available (from the companies: Merck, Darmstadt; Riedel de Haen, Seelze; Roth, Karlsruhe or Serva, Heidelberg, all Germany).

Cell line

The human pre-B cell line Nalm6 was a gift from S. J. Korsmeyer (Metabolism Branch, National Cancer Institute, Bethesda, Maryland 20205, U.S.A.) and grown in RPMI 1640 medium supplemented with 4 mM L-glutamine (both Gibco, Eggenstein, Germany) and 10% fetal calf serum (Seromed/Biochrom, Berlin, Germany). Every 3 months, the Nalm6 cells were tested for mycoplasms and surface antigens (HLA-DR, CD10, CD19 and CD22 positive; CD5 and CD20 negative). The volume of Nalm6 cells was determined in a Schaerfe system cell counter (Schaerfe, Reutlingen, Germany) by H. R. Scherf at the German Cancer Research Centre.

Incubations

Exponentially growing Nalm6 cells were harvested by centrifugation (500*g*, 10 min, room temperature), suspended in fresh RPMI 1640 medium containing 10% fetal calf serum, seeded into 12 well culture dishes at a density of 10^7 cells/2 ml and incubated with ^{3}H-MTX as indicated in the figures. Blasts isolated from patients were incubated under the same conditions.

At the times indicated in the figures, aliquots of 10^7 cells were analysed for synthesized ^{3}H-MTXPG. Cell suspensions were added to ice cold PBSS, washed twice in PBSS to remove extracellular ^{3}H-MTX, and viability was determined by trypan blue exclusion. For isolation and quantitation of methotrexate and its metabolites, the method of Kamen and Winick [22] was further optimised. After the last wash, the cell pellet was suspended in 800 µl lysis buffer (10 mM Tris, 5 mM EDTA and 150 mM mercaptoethanol adjusted to pH 8.0 with HCl). As an internal standard, 25 µl of a solution of the nine MTX metabolites (MTXPG1-7, 7OH-MTX and 2,4-diamino-N^{10}-methylpteroic acid) were added and radioactivity determined in 50 µl of cell lysate. The lysate was boiled for 10 min and cellular debris removed by centrifugation at 4500*g* for 1 h at 4°C. After adding 2 ml methanol to the supernatant, the probe was stored in ice for at least 30 min, again centrifuged (4500*g*, 15 min, 4°C) and the supernatant evaporated to dryness.

In experiments in which MTX-efflux was followed, 10^7 cells incubated with ^{3}H-MTX were collected by centrifugation, washed twice in prewarmed complete RPMI medium and resuspended at the same cell density in fresh complete medium (without ^{3}H-MTX) for the periods indicated in the figures. These cells were then washed to remove extracellular ^{3}H-MTX and processed as described above.

Analysis of ^{3}H-MTXPG

The residue after evaporation was dissolved in 200 µl water for HPLC analysis performed with a Waters/Millipore HPLC system consisting of a gradient former, two pumps (510 and 6000A), LC-spectrophotometer Lambda Max Model 481 and an Integrator 740 data module. Cell extracts were analysed by HPLC using a modification of the method of Jolivet and Schilsky [23] and Kamen and Winick [22]. Briefly; 125–150 µl of the probe were injected on to an ODS Ultrasphere 5 µ; 4.6 × 250 mm column with a precolumn Hypersil ODS 5 µ; 20 × 4 mm (both Beckmann, München, Germany) and MTXPG eluted by a mobile

phase consisting of 5 mM tetrabutyl-ammonium-phosphate pH 7.4/acetonitrile; 80%/20% (v/v) with the following linear gradient: 20% acetonitrile for 5 min increasing to 56% acetonitrile by 75 min, with a flow rate of 1.5 ml/min. The eluate was monitored at 300 nm, 0.75 ml fractions were collected, 3 ml of scintillation fluid (Ultima Gold XR Packard) added and radioactivity analysed using a TRI Carb 2000 (Canberra Packard) liquid scintillation counter. This method allows the separation of all known MTX metabolites from cellular debris and from each other.

RESULTS

Figure 1 shows HPLC-separation of patient lymphoblasts incubated for 24 h with ^{3}H-MTX. The recovery of the radioactivity from cell lysis to HPLC was between 85 and 95%. The detection limit of a single ^{3}H-MTX metabolite was 0.01 pmol per 6×10^6 analysed cells. ^{3}H-2,4-diamino-N^{10}-methylpteroic acid and ^{3}H-7OH-methotrexate as metabolites of ^{3}H-MTX were not detected in any incubation of Nalm6 cells or patient blasts with ^{3}H-MTX. The radioactive peaks eluting before ^{3}H-MTXPG2 and ^{3}H-MTXPG3 were not characterised.

Nalm6 cells

Figure 2 shows the concentration dependence of ^{3}H-MTX uptake, and synthesis of ^{3}H-MTXPG in Nalm6 cells. After 24 h incubation with different concentrations of ^{3}H-MTX between 2.05 and 5.32 pmol/10^6 cells of total ^{3}H-MTXPG were detected. Saturation was reached between 0.65 and 0.9 μM ^{3}H-MTX. The distribution of ^{3}H-MTX among the different polyglutamates is constant and independent of ^{3}H-MTX concentrations, with MTXPG3 as the main metabolite.

Since plasma MTX-levels during low-dose oral MTX-therapy are approximately 1 μM MTX [18, 24] and most ^{3}H-MTXPG were formed in incubations with 0.9 μM ^{3}H-MTX, all further experiments were performed with 1 μM ^{3}H-MTX. Because of manufacturing problems of Amersham, ^{3}H-MTX from Moravek Biochemicals was used for the following experiments.

The time dependence of ^{3}H-MTX uptake and ^{3}H-MTXPG formation in Nalm6 cells is shown in Figure 3. The maximum amount of total ^{3}H-MTXPG was synthesised after 15 h incubation with 1 μM ^{3}H-MTX, with a plateau up to 24 h. ^{3}H-MTX and ^{3}H-MTXPG2 rapidly attained a steady state level of about 0.2 pmol and

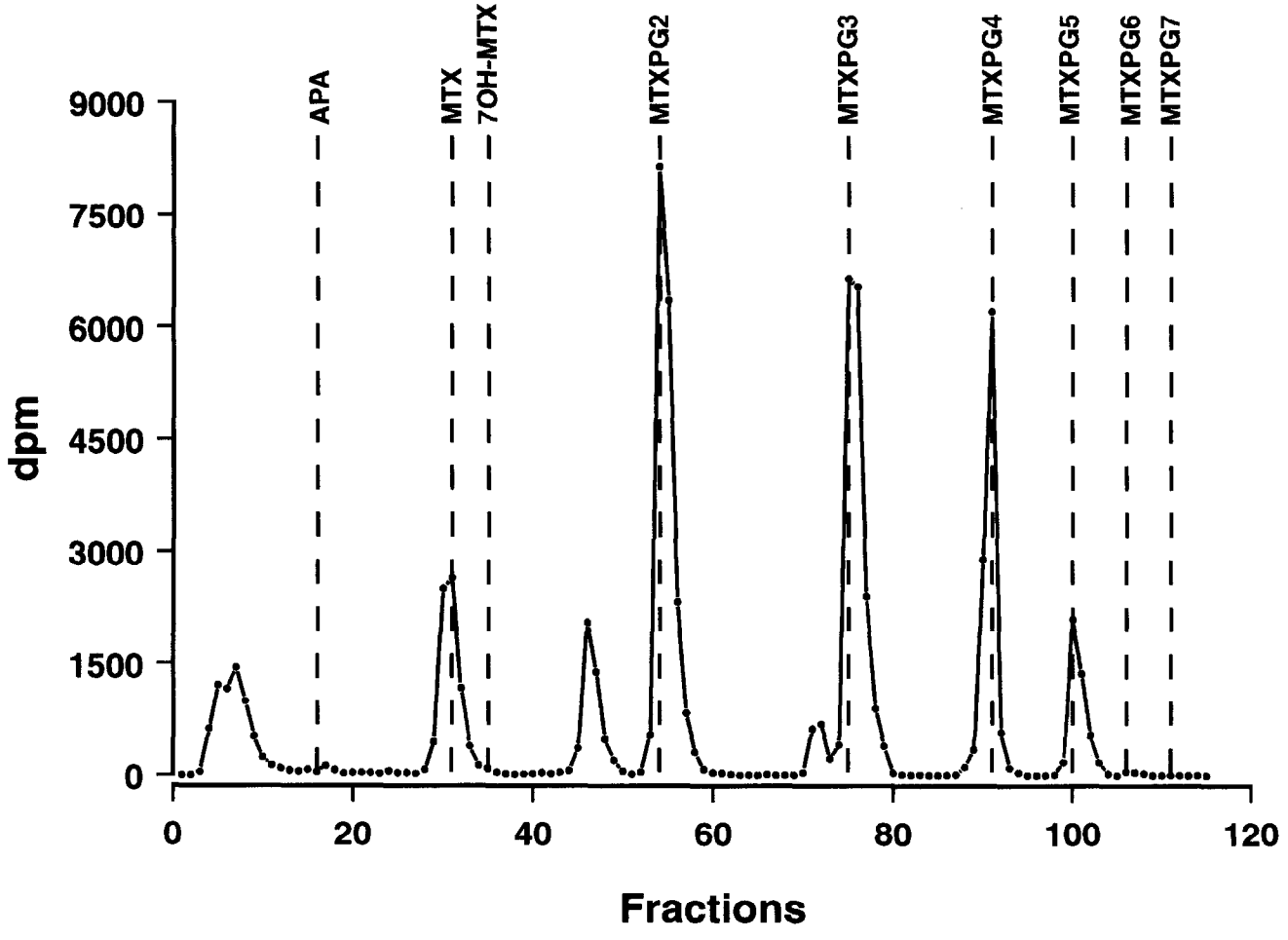

Figure 1. Separation of ^{3}H-MTXPG in lymphoblasts from a patient. Cells (10^7) were incubated with 1 μM ^{3}H-MTX (Moravek) for 24 h and the ^{3}H-MTXPG isolated as described in Materials and Methods. After addition of MTXPG standards an aliquot of the sample was injected onto the HPLC column; 0.75 ml fractions were collected and counted for radioactivity. The solid line represents the amount of radioactivity in each fraction, the hatched lines the retention times of the different methotrexate metabolite standards.

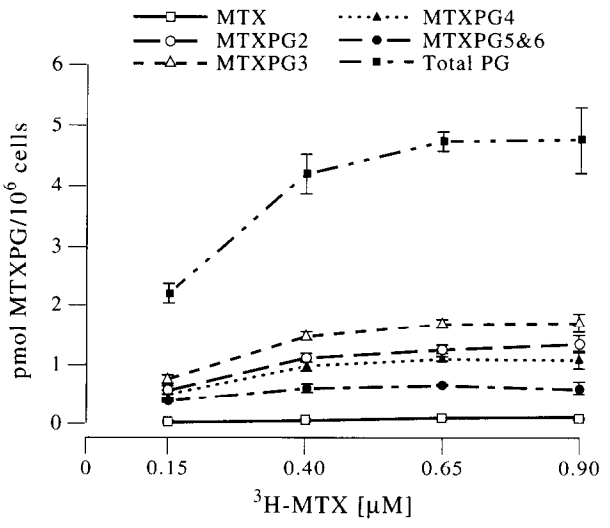

Figure 2. ^{3}H-MTXPG in Nalm6 cells. Cells (10^7) per duplicate were incubated with either 0.15, 0.4, 0.65 or 0.9 μM ^{3}H-MTX (Amersham) for 24 h, harvested and ^{3}H-MTXPG isolated, separated and quantified as described in Materials and Methods.

0.4 pmol/10^6 cells, respectively, while elongation to the longer chain ^{3}H-MTXPG3-5 occurred continuously. After 3 h of incubation, ^{3}H-MTXPG3 was the predominant methotrexate polyglutamate, but from 6 to 24 h incubation ^{3}H-MTXPG3, 4 and 5 were found in similar high amounts (each around 30% of total ^{3}H-MTXPG). MTXPG6 was only found at very low levels.

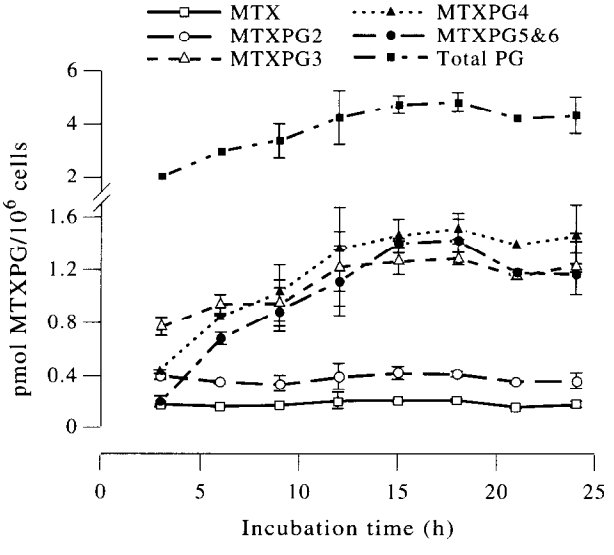

Figure 3. Time-dependent formation of ^{3}H-MTXPG in Nalm6 cells. Nalm6 cells (10^7) in 2 ml per well were incubated with 1 μM ^{3}H-MTX (Moravek). Three wells were analysed per time point. After the times indicated, cells were harvested, viability checked by trypan blue exclusion and ^{3}H-MTXPG isolated and analysed as described in Materials and Methods. Each point is the mean with standard deviations of three incubations.

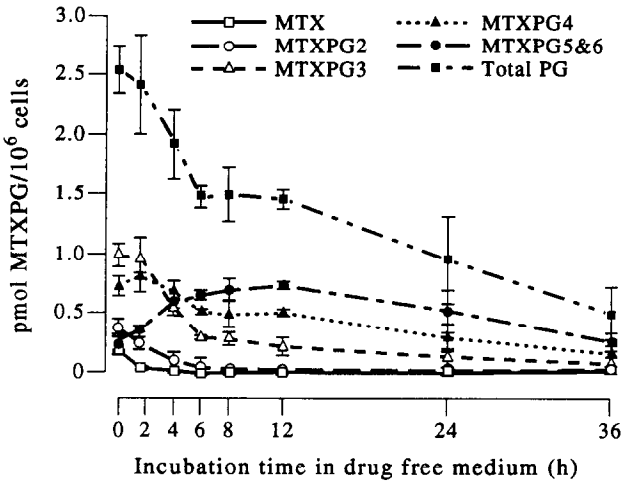

Figure 4. ^{3}H-MTXPG persistence. For these experiments Nalm6 cells were incubated in flasks in 2 × 27 ml RPMI medium at a density of 5×10^6 cells/ml with 1 μM ^{3}H-MTX (Moravek) for 15 h. Three aliquots of 10^7 cells were then harvested and analysed for viability and ^{3}H-MTXPG as described in Materials and Methods. The remaining cells were washed twice in MTX-free medium, suspended at a density of 10^7 cells/2 ml and seeded into wells. At the incubation times indicated the cells were harvested, ^{3}H-MTXPG isolated and quantified in each of the wells. Two experiments, one in triplicate one in duplicate are superimposed in this figure, each point is the mean with standard deviation of five incubations.

For efflux experiments, cells were incubated with 1 μM ^{3}H-MTX in a total volume of 27 ml at a density of 5×10^6cells/ml in 250 ml culture flasks. After 15 h, cells were washed in medium and transferred to 12 well dishes at a cell density of 10^7/2 ml. Two individual experiments, one in triplicate and one in duplicate, are superimposed into one figure (Figure 4). ^{3}H-MTXPGs showed a biphasic decrease, a more rapid phase to 60% of the original value of the loaded cells after 8 h and a slow phase to 30% of the original value after 24 h incubation in MTX-free medium.

MTX and MTXPG2 rapidly reached a low and, in the case of MTX after 8 h, non-detectable intracellular level. MTXPG3 and MTXPG4 were stable up to 2 h after washing, and then decreased slowly to 36 h, while MTXPG5 was still formed from precursors up to 12 h after transfer into MTX-free medium, and then decreased very slowly. Between 8 h and 36 h after loading cells with ^{3}H-MTX, the amount of each persisting polyglutamate was proportional to its chain length. MTXPG6 showed a very slight increase to 0.04 pmol between 6–8 h.

An observation we made is that the geometry of the culture dish in which cells were incubated with ^{3}H-MTX influences the uptake of MTX and the distribution of radioactivity among MTXPG in the cells. Cells in wells in 2 ml medium accumulated between 4 and 5 pmol/10^6 cells MTXPG with MTXPG3, 4 and 5 as the major metabolites after 15 h, while cells incubated in flasks took up 2.5 pmol/10^6 cells with MTXPG3 as the main metabolite (Figures 3 and 4). In wells, the ratio, surface to height, was 7.5 and in flasks 16.5. In wells 10^7 cells covered a surface of 3.89 cm^2, while in flasks only 1.54 cm^2 was covered. For the efflux experiments, the cells were transferred from flasks to wells and the amount of MTXPG5 increased. This effect is

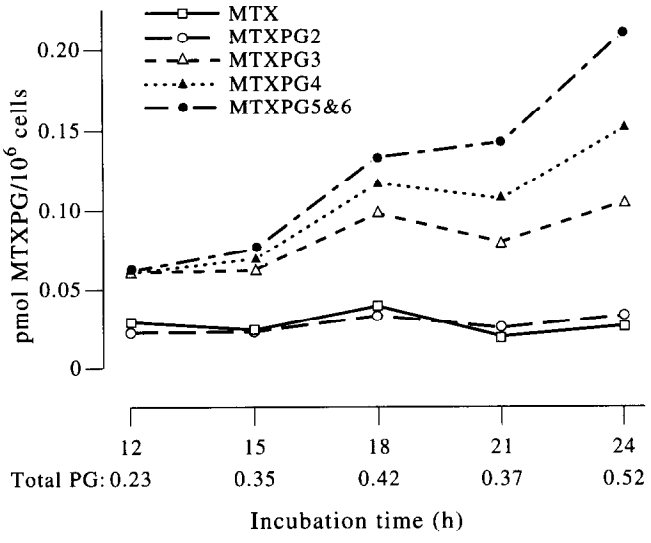

Figure 5. Time-dependent formation of ^{3}H-MTXPG in lymphoblasts of a patient with c-ALL. Five wells with 10^7 lymphoblasts in 2 ml were incubated with 1 μM ^{3}H-MTX (Moravek). For each time point, lymphoblasts were harvested, viability checked and ^{3}H-MTXPG isolated and analysed as described in Materials and Methods. The amounts of total MTXPG are indicated as pmol/10^6 cells along the *x*-axis at the different time points.

highly reproducible, as is reflected in the two experiments performed in the space of one year by two different people superimposed for Figure 4. Since MTX uptake and MTXPG formation depend on energy, the availability of nutrients in the immediate vicinity of the cells is probably rate limiting.

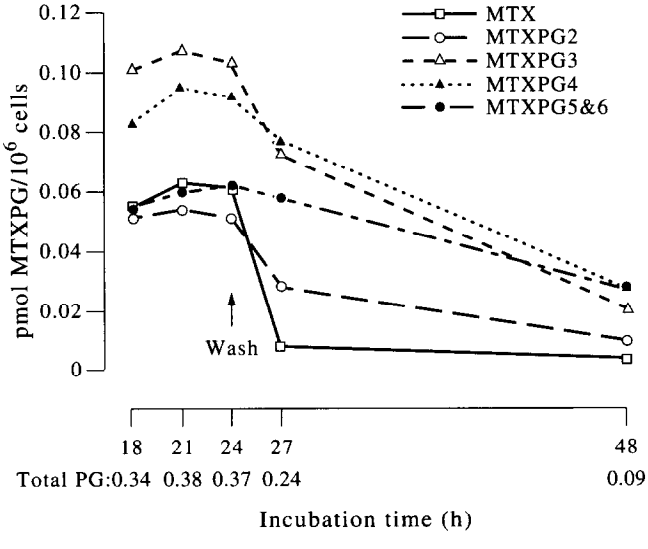

Figure 6. Time-dependent formation and degradation of ^{3}H-MTXPG in lymphoblasts of a patient with T-ALL. Five wells with 10^7 lymphoblasts in 2 ml each were incubated with 1 μM ^{3}H-MTX (Moravek). At each time point up to 24 h one well was harvested, viability checked and ^{3}H-MTXPG isolated and analysed as described in Materials and Methods. After 24 h the blasts remaining in the two wells were washed twice in MTX-free RPMI medium and aliquots of 10^7 cells incubated in 2 ml medium for a further 3 h and 24 h without MTX. The amounts of total MTXPG are indicated as pmol/10^6 cells along the *x*-axis at the different time points.

Patient blasts

With the method established, and the kinetics of MTXPG formation and persistence in Nalm6 cells known, we ran experiments with patient blasts. Time dependence of ^{3}H-MTXPG formation in lymphoblasts from 2 patients was tested (Figures 5 and 6). In one patient, the kinetics of ^{3}H-MTXPG formation (Figure 5), was measured, and in the other MTXPG persistence in MTX-free medium (Figure 6) was also analysed. Such detailed experiments are usually restricted by the number of patient lymphoblasts.

In lymphoblasts from both patients, the amount of ^{3}H-MTXPG synthesised was only about one tenth of that found in Nalm6 cells after the same incubation time with ^{3}H-MTX.

Lymphoblasts from the patient suffering from c-ALL (Figure 5) continued synthesis of ^{3}H-MTXPG3, 4 and 5 up to 24 h (the last point determined). ^{3}H-MTX and ^{3}H-MTXPG2 were at a steady state level of 0.02–0.06 pmol in patient lymphoblasts, similar to the levels in Nalm6 cells. At each time point analysed, ^{3}H-MTXPG5 followed by ^{3}H-MTXPG4 and ^{3}H-MTXPG3 were the predominant polyglutamates in lymphoblasts of the c-ALL patient.

^{3}H-MTXPG6 was lower than 0.01 pmol at each time point and was added to the value for ^{3}H-MTXPG5.

In lymphoblasts of the T-ALL patient high amounts of ^{3}H-MTXPG3 and ^{3}H-MTXPG4 were found at all time points in the presence of ^{3}H-MTX. After 3 h and 24 h incubation in MTX-free medium, the total amount of ^{3}H-MTXPG declined to 66% and 24%, respectively of the ^{3}H-MTX loaded cells. During the first 3 h in MTX-free medium ^{3}H-MTX decreased rapidly, the levels of the other MTXPG decreased more slowly according to the chain length up to ^{3}H-MTXPG5, which stayed at approximately the same level as in the presence of ^{3}H-MTX. After 24 h incubation in MTX-free medium, ^{3}H-MTXPG4 and 5 were the predominant ^{3}H-MTXPG.

DISCUSSION

The sample preparation described by Jolivet and Schilsky [23] and Kamen and Winick [22] was simplified because, in our system using ^{3}H-MTX, no contaminating background as in the radioligand assay by Kamen and Winick [22] was expected. We found the recovery of total ^{3}H-MTXPG, based on radioactivity of the lysate in the Tris-mercapto-ethanol-EDTA buffer, to be >90%. Therefore, sonication and DEAE-cellulose separation was omitted. The original HPLC method of Jolivet and Schilsky [23] was modified and allowed a separation of all known MTX metabolites in our HPLC method, i.e. 2,4-diamino-N^{10}-methylpteroic acid, 7OH-MTX and MTXPG1-7. Previously, only methods separating MTX from 7OH-MTX [21, 25] or MTX from the other MTXPG [23, 26] had been used. We monitored the eluent at 300 nm rather than 254 nm because MTX, MTXPG and 7OH-MTX show a maximum at 300 nm [21]. Another important modification to the methodology of Whitehead and colleagues [15, 17] and Lin and colleagues [16] is the use of an isotonic wash containing protein to remove extracellular ^{3}H-MTX effectively from cell surfaces after incubating the cells with ^{3}H-MTX,

because MTX has a high protein binding activity [26]. With PBS containing 1% fetal calf serum or 0.1% bovine serum albumin, high artefactual MTX levels were completely eliminated and no efflux time to lose 'exchangeable MTX' (Whitehead and colleagues [15]) is needed. The high MTX levels observed by other authors are probably due to extracellular MTX. Our results also show that degradation and efflux of MTX occurs quite rapidly in MTX-free medium at 37°C. A rapid processing of blasts at 4°C (efflux is energy dependent, see below) is, therefore, important to achieve a true measure of ^{3}H-MTX and ^{3}H-MTXPG accumulation in the presence of ^{3}H-MTX.

We observed a conspicuous difference in MTX uptake and the pattern of MTXPG formed when ^{3}H-MTX of greater (Moravek 98%) or lesser purity (Amersham 75%) were compared. A paper by Kamen and colleagues [27] described the preferential uptake of impurities in the ^{3}H-MTX preparations, namely [^{3}H]-*p*-amino benzoylglutamate, by a MTX-resistant L1210 subline. Since we analysed the MTX metabolites by HPLC and not as bulk radioactivity, we thought we could ignore the impurities, all the more so, since the authors observed that the uptake of ^{3}H-MTX was not influenced by the impurity. However, apparently there was/were impurity(ies) which inhibit the folylpolyglutamate synthase leading to shorter chain MTXPG in incubations with Amersham's ^{3}H-MTX where MTXPG3 is the dominant metabolite (Figure 2), while with Moravek's ^{3}H-MTX, MTXPG3, 4 and 5 were formed at equal amounts (Figure 3).

The intracellular concentration of MTXPG in Nalm6 cells after 24 h amounted to 5 pmol/10^6 cells. Nalm6 cells have a median diameter of 10.4 μm, so 10^6 cells therefore represent a volume of 0.6 μl, which amounts to an intracellular concentration of 8 μM MTXPG. This is an 8-fold increase over the extracellular concentration of MTX, i.e. the uptake of MTX occurs against a concentration gradient.

MTXPGs are taken up from the cytoplasm into lysosomes by facilitated transport, degraded by thiol-dependent folylpolyglutamate hydrolase to MTX [28]. MTX is then exported into the cytoplasm and from there into the extracellular space by two different pumps [29, 30]. In Nalm6 cells and in patient blasts, the complex kinetics of the synthesis of longer chain MTXPG from MTX, of MTXPG degradation, and MTX efflux in the absence of extracellular MTX, are reflected in the shapes of the curves of the individual MTXPG. The slight decrease in MTXPG5 and 6 and total MTXPG observed after 21 h in Nalm6 cells, even in the presence of MTX, is probably due to the start of MTXPG degradation, since MTXPG with longer chains are better substrates for the facilitated intralysosomal transport and better substrates for folylpolyglutamate hydrolase than short chain MTXPG, whereby the transport is the rate-limiting step [28].

The ability of patient blasts to synthesise long chain MTXPG has been recognised as a prognostic factor for successful treatment, although discrepancies grow with the data accumulated [15–18, 24]. In addition to the synthesis, surely the persistence and continued elongation of MTXPG in the absence of extracellular MTX should reflect sensitivity of patient blasts against this agent.

In the c-ALL patient, MTXPG5 was at all times the predominant polyglutamate, while in the T-ALL patient MTXPG5 only dominated after efflux. Such MTXPG patterns were also found by others comparing ALL with acute non-lymphoblastic leukaemia [16]. The concentrations of MTX we found in patient blasts are comparable with those observed in blasts isolated from patients 44 h after low-dose MTX [18], and also to those in blasts incubated *in vitro* with MTX [17]. The amount of MTXPG found in patient blasts is about one-tenth of that in Nalm6 cells, and still the method allows easy detection of all MTX metabolites after efflux. The lower MTX uptake in fresh blasts compared to Nalm6 cells is caused by various factors. The fresh blasts do not grow in culture, while Nalm6 cells were in the logarithmic phase of their growth at the time of MTX addition, and MTX uptake is highest in the S-phase of the cell cycle. The amount of MTX taken up by fresh blasts is probably also a measure of their MTX-sensitivity and, therefore, varies among patients as will be shown in our ongoing study in cooperation with three childrens' hospitals. The study will also show the validity of examining MTX uptake, MTXPG synthesis and persistence in fresh patient blasts as prognostic factors for therapy.

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